GENE EXPRESSION ANALYSIS OF CELLULAR CHANGES IN RESPONSE TO FIBROBLAST INJECTION AND WOUNDING

by
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A thesis submitted to Johns Hopkins University in conformity with the requirements for

the degree of Master of Science

Baltimore, Maryland
April 2021

Abstract

Within the United States, there are over two million individuals who have undergone amputations with an additional 185,000 receiving amputations each year (Yang et al.). Amongst the amputee population, 48.2% of individuals reported experiencing skin dermatoses at the amputation site that prevented the use of their prosthetics (Yang et al.). In an attempt to solve this problem and increase the ease of use of prosthetics, one possible solution has been modification of the epidermal skin identity at the stump site. The skin of the limbs is thin and prone to injury, but by turning this thin skin into tougher volar (palmoplantar) skin, prosthetic use would become analogous to putting on a shoe. One method for this has involved the injection of volar dermal fibroblasts into the dermis of thin skin, resulting in a multitude of phenotypic changes. What is still unknown is what changes are occurring on the cellular level and the mechanism by which these changes take place. Little is known about how gene expression changes following injection. In order to elucidate the mechanism by which epidermal identity can be altered, it is important to understand how gene expression changes following injection. To investigate the changes in gene expression, RNAseq analysis was performed using biopsies from injected samples, and compared to a vehicle-injected control and native volar skin. clusterProfiler was used to analyze the changes in gene expression, the functions each gene was associated with, and which genes tended to be grouped together. Alterations to gene expression during wound healing were of similar interest, and similar methodology was used to investigate gene expression at different locations on or near a wound.

Primary Reader and Advisor: Luis A. Garza

Secondary Reader: Robert Horner

Preface

This thesis is the combination of my research on the epidermis and dermis by fibroblast injections (Part 1) and an additional project on wound healing in the Garza Lab (Part 2). This thesis is finished in April 2021. The research was done in the Department of Dermatology at the Johns Hopkins School of Medicine in Baltimore, Maryland. I was introduced to this project by Dr. Sam (Seakwoo) Lee in 2019, and have been working under his guidance since. Dr. Lee has been an amazing mentor to me and has provided much assistance in teaching my laboratory techniques and bioinformatics.

I would like to thank my PI, Dr. Luis A. Garza and our lab manager, Dr. Sam (Seakwoo) Lee, for their mentorship throughout both my undergraduate and graduate years. Dr. Garza has broadened my knowledge and helped me to understand the context of my work and appreciate the complexity of the subject. He has been an enormous help in interpreting my results and suggesting new directions over the past year. Dr. Lee has also been of great assistance in helping me to develop the skills necessary for this project and providing assistance when needed. I would also like to thank Chenyi Lyu and Evan Sweran from my lab for their continued support and suggestions.

I would also like to thank my professor, Dr. Anna Coppola, and my advisor, Dr. Robert Horner, for their guidance.

Finally, I'd like to thank the other students in the program, my family, and my friends for their continuous support throughout the program.

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Chapter 1: A Review of Epidermal Differentiation and Wound Healing

1.1 Amputations in the United States

Within the United States, there are slightly over two million patients who have undergone amputation . An additional 185,000 individuals receive amputations in the United States each year (Ziegler-Graham et al.). Amongst the veteran population with amputations, 48.2% of individuals reported experiencing skin breakdown or rash at the amputation site within the past year (Table 1, Yang et al.). Of these individuals, 5.9% reported experiencing these problems all the time, while 25.2% reported the problem occurring more than 50% of the time (Yang et al.). Additional skin issues were described, including staphylococcus infection and sores, scar reopening, abrasions, blisters, and sores.

Table 1: Prevalence of Skin Dermatoses in Veterans (Yang et al.).

How often do you experience any skin problems at the stump site?	Rarely (<10%)	22.7%
	Sometimes(10%-50%)	46.2%
	Often (>50%)	25.2%
	Always	5.9%
Description of cause of problems	Prosthesis	68.7%
	Heat-related	17.9%
	Stump/bone complications	7.5%
	Subsequent related operations	6.0%

Skin dermatoses at the amputation site are not a problem that goes away. The prevalence described above was reported by amputees at least 38 years following their amputation (Yang et

al.). Perhaps most importantly, these issues can interfere with the use of prosthetics. Of the respondents who experienced dermatoses, 68.7% described their prosthetic as the cause of their problems. Additionally, 55.6% reported having limited ability to use their prosthetic as a result of skin problems (Yang et al.). 37.1% of amputees had altered or replaced their prosthetic due to skin problems (Yang et al.). Dermatologists and researchers believe that the majority of skin dermatoses are caused by frictional and mechanical trauma by the prosthesis (Dudek et al.). Current treatment methods and therapies are not able to adequately address the root of this problem, but regenerative medicine, such as epidermal identity modification, may be able to.

1.2 Epidermal Skin Identity

The epidermis is a highly complex organ that is not the same throughout all areas of the body. The most significant differences can be observed between volar skin, found on the palms and soles, and nonvolar skin, found on the rest of the body. Some variation exists within nonvolar skin, with the cellular epidermal thickness ranging from 45.1 to 68.1 microns at the dorsal forearm (Sandby-Møller et al.). The total epidermal thickness at this site ranges from 62.2 to 87.6 microns including the stratum corneum, while the shoulder and buttock have somewhat thicker epidermal layers (Sandby-Møller et al.). A much greater difference exists between volar and nonvolar skin. The average skin thickness of the fingertips, a volar location, ranged from 98-111 microns, with males having slightly thicker epidermis (Fruhstorfer et al.). Based on this information, the volar epidermis can be nearly twice as thick than nonvolar epidermis, depending on the individual and the body site. Of additional interest is the stratum corneum, the most superficial layer of dead cells. This layer measures between 11.0-18.3 microns at nonvolar sites, and between 130 to 795 microns at volar sites (Sandby-Møller et al., Fruhstorfer et al.). With the

exception of the basal stratum, layers of the epidermis are typically thicker in volar skin (Vela-Romera et al.)

Additional differences exist on the cellular level. Cells in the volar epidermis are more abundant and larger than cells in the nonvolar epidermis (Vela-Romera et al.). This is particularly true of the keratinocytes of the spinosum and granulosum, which may be up to twice as large in volar tissue. Variations in cell type are also present between skin sites. Corneocytes, a type of terminally differentiated keratinocyte, have significantly higher abundance in volar tissue (Vela-Romera et al.). Other keratinocytes and Merkel cells are more abundant in the volar epidermis, while melanocytes and Langerhans cells are more abundant in the nonvolar epidermis (Vela-Romera et al.).

1.3 Epidermal Skin Markers

Various markers exist that show differential expression in the volar and nonvolar epidermis. Cytokeratin 5/6, also known as KRT5 is one such marker. KRT5 is strongly expressed in the basal layer of both volar and nonvolar skin, but in the suprabasal layers, it is expressed to a greater extent in volar tissues (Vela-Romera et al.). Due to its high levels of expression in the basal layer, KRT5 is an excellent marker for basal keratinocytes, as is KRT14. Involucrin (IVL) exhibits the opposite pattern, being expressed more in the suprabasal layers of the nonvolar epidermis (Vela-Romera et al.). Alongside KRT1 and KRT10, IVL is a marker for suprabasal keratinocytes. Similar trends can be seen with dickkopf 1 and 3, with DKK1 being expressed to a greater extent in volar skin and DKK3 being expressed more in nonvolar skin (Yamaguchi et al.). In fact, increased expression of DKK1 is involved in the inhibition of melanocyte growth, a contributing factor to the lower number of melanocytes observed in volar skin (Yamaguchi et al.).

Another important marker is keratin 9, which is expressed at significantly higher levels in the volar epidermis. Like CK5/6, KRT9 is also most differentially expressed in the suprabasal layers. KRT9 is necessary for differentiation of the volar epidermis, and plays a large role in the maintenance of epidermal integrity (Fu et al.). KRT9 knockdown mice are more prone to developing calluses, and expression patterns of other keratins are disrupted in the absence of KRT9 (Fu et al.). KRT9 is expressed in keratinocytes, while this expression is sustained by volar fibroblasts (Liao et al.). Expression of KRT9 can be inhibited by DDX58, a receptor for ds-RNA, indicating that ds-RNA sensing may play a role in skin thickness (Liao et al.). When cultured alone, volar keratinocytes, but not fibroblasts, continue to produce KRT9, while nonvolar keratinocytes rarely express KRT9 alone (Yamaguchi et al.). KRT9 is an especially interesting gene, as it lays the groundwork for epidermal transplant therapies. By taking volar fibroblasts and culturing them with nonvolar fibroblasts, researchers were able to induce KRT9 expression in the nonvolar fibroblasts (Yamaguchi et al.). Even before this study, researchers had seen that transplanted skin fibroblasts were capable of releasing vectors for at least 8.5 months, although the expression decreased drastically within one month (Palmer et al.).

1.4 Fibroblasts

As demonstrated by the experiment mentioned above, fibroblasts have the ability to modify the environment around them. Fibroblasts are a very diverse cell population, as they are present throughout a wide variety of tissues in the body. The primary purpose of fibroblasts is to establish and maintain the stroma of the body (Sorrell & Caplan). This is largely done through the production of the extracellular matrix, which is crucial for the development and maintenance of organs (Sorrell & Caplan). In general, fibroblasts may have multiple embryonic origins depending on which tissue they are found in, but within the skin, fibroblasts may derive from

neural crest cells or the dermomyotome. Depending on environmental conditions, dermal fibroblasts have also demonstrated the ability to differentiate along adipogenic, osteogenic, chondrogenic, and myogenic lineages (Lorenz et al.). Multiple signals that interact with fibroblasts are involved in the development of skin, including Wnt1 and Msx1 (Sorrell & Caplan). Fibroblasts are not only important in the development of skin: they are also important for skin maintenance, healing, and normal function.

1.5 Fibroblast Function in Skin

Fibroblasts have many vital roles in the skin. One essential function of fibroblasts is the interaction with keratinocytes. Fibroblasts and keratinocytes are capable of forming a paracrine loop in response to wounding (Sorrell & Caplan). They are also capable of regulating keratinocyte physiology through the production of factors such as GM-CSF (Sorrell & Caplan). Keratinocytes are also able to induce the expression of TGF-β in fibroblasts, which will then increase laminin and collagen production in keratinocytes (Sorrell & Caplan). Fibroblasts are also involved in the formation of the basement membrane underneath the dermis. Through the regulation of collagen in epidermal cells, they are able to assist in the construction and organization of the basement membrane (Sorrell & Caplan). Finally, fibroblasts play a large role in epidermal cell differentiation. When added to the reticular dermis, fibroblasts promote keratinocyte growth and attachment (Krejci et al.). Even when transplanted from different locations, fibroblasts may differentially interact with other cells, leading to different tissue development and differentiation (Sorrell & Caplan).

In addition to their role in epidermal development, fibroblasts are vital to the wound healing process. While many different cytokines and growth factors are involved in regeneration, fibroblast growth factor (FGF) is one of the more important and better understood proteins.

Throughout the body, FGF is capable of regulating the expression of other growth factors, and has a heavy influence on cell migration, differentiation, and proliferation (Maddaluno et al.). Fibroblasts in granulation tissue, dermal tissue, and epidermal γδ T cells produce FGF7 in response to wound healing. FGF7 then stimulates keratinocyte migration through the activation of the FGFR2B receptor protein, although there appears to be redundancy with other FGFs as well (Maddaluno et al.). Increased FGF expression increases the rate at which epidermal wounds heal, and increased the vascularization of wounded tissue by promoting angiogenesis (Maddaluno et al.). FGFR knockdown mice demonstrate impaired wound contraction and re-epithelialization, indicating that FGFs are necessary for these wound healing processes (Maddaluno et al.). This interaction between fibroblasts and keratinocytes is essential for proper skin repair following wounding.

1.6 Wound Healing Process

Wound healing is a complex process by which connective tissues are repaired and the tissue returns to normal function without need for additional treatment. Primary wound healing occurs when the majority of epithelial cells survive, but the basement membrane is disrupted (Enoch & Leaper). Epithelial regeneration follows, and the wound closes within 24 hours (Enoch & Leaper). Primary healing may be delayed in the wound has been contaminated, but still proceeds normally. Secondary healing is the mechanism by which severe wounds heal, and relies on epithelialization and wound contraction (Enoch & Leaper). The majority of acute wounds recover via primary healing.

Wound healing occurs in three overlapping phases: inflammation, tissue formation, and tissue remodeling. During inflammation, monocytes bind to extracellular membrane proteins, triggering their transformation into macrophages (Singer & Clark). Macrophages appear to play

a vital role in proper wound healing, as elimination of macrophages results in delayed neutrophil activity and decelerated fibroblast proliferation (Leibovich & Ross). Epithelialization begins shortly after injury, during which cells migrate and begin to proliferate (Singer & Clark). Granulation tissue begins to fill the wound four days following injury as fibroblasts deposit large amounts of collagen to create a provisional matrix for tissue to develop (Singer & Clark). Finally, the new tissue undergoes remodeling, turning into a scar as collagens cross-link or are degraded (Singer & Clark).

1.7 Pathways in Wound Healing

A variety of molecules and pathways are involved in wound healing. One of the essential pathways for wound healing is the Notch pathway, involved in cell fate determination. The binding of Jag protein to the Notch receptor triggers the differentiation epidermal stem cells into various lineages (Yang et al.). The Notch pathway may be involved in regulated cell adhesion, thus altering the signals received by stem cells and allowing them to proliferate (Yang et al.). The Jag protein also seems to be involved in linking the Notch pathway to the Wnt pathway, although the mechanism is unclear.

Wnt protein is capable of regulating cell proliferation, differentiation, and migration through interactions with the Frz receptor (Yang et al.). High levels of Wnt signaling seem to be capable of triggering hair structure development by epidermal stem cells (Yang et al.). The activation of the Wnt/β-catenin is heavily involved in wound healing, and is capable of modifying epithelial tissue patterning to improve wound healing (Fathke et al.).

Chapter 2: Gene Expression Modifications Following Dermal Fibroblast Injection

2.1 Introduction

Fibroblasts are a heterogenous cell type found in a variety of tissues throughout the body. Dermal fibroblasts are well-known for synthesizing components of the extracellular matrix, but they are also highly involved in cell proliferation and migration (Wong et al.). Fibroblasts interact with a large number of cells in the skin, including keratinocytes, vascular endothelial cells, and hair follicles (Sorrell & Caplan). Given their ability to interact with diverse cell types and modify their environment, fibroblasts would be expected to be capable of altering their environment when transplanted. This is largely due to the remarkable positional memory of fibroblasts. Fibroblasts exhibit clear topographic differentiation, with many HOX genes being differentially expressed across tissue types (Chang et al.) The HOX genes expressed in adult fibroblasts are similar to the expression patterns established in development, further demonstrating the positional memory of fibroblasts (Chang et al.). Once moved to a new location, fibroblasts should begin to alter their new environment to be more similar to their old one. This has already been observed in other experiments: papilla cells, a fibroblast-like cell type, are capable of stimulating the development of new hair follicles when implanted into existing follicles (Jahoda et al.). This pattern should be observable following the injection of dermal fibroblasts from one skin location to another, and would be measured by changes to the epidermal and dermal layers.

Fibroblast transplantations have been used in multiple therapies. Within various tissue types, fibroblasts have been shown to have regenerative properties. Treatment of vocal fold scars of the larynx with fibroblast injections results in rapid healing (Chhetri & Berke). Due to their ability to produce the extracellular matrix, fibroblast injections in the skin have been

capable of correcting epidermolysis bullosa, a condition in which the skin blisters easily (Ortiz-Urda et al.). Fibroblasts have also been used to treat fine lines and wrinkles, with injections being effective after six months with minimal adverse effects (Tang et al.). Of additional interest it the use of fibroblasts in wound healing. Injection of dermal fibroblasts are irradiated sites has been shown to be beneficial for the healing process. Injections were capable of increasing the skin breaking load, tensile strength, elasticity, and toughness at wounded locations. (Ferguson et al.).

Due to the high prevalence of skin dermatoses at amputation sites, it would be beneficial to develop new treatment methods capable of preventing the development of such dermatoses (Yang et al.). Application of autologous fibroblasts appears to be a promising treatment option. The aim of this therapy is to "volarize" the skin of the stump site, allowing it to become phenotypically similar to the palms and soles (Thangapazham et al.). Human fibroblasts injected in mice have been shown to be capable of surviving for at least eight weeks, meaning that fibroblast injection treatments are a feasible model (Thangapazham et al.). Following injection, fibroblasts are initially tightly clustered, but disperse throughout the tissue over the course of four to eight weeks. (Thangapazham et al.).

Ongoing research by the Garza Lab has indicated that injection of dermal fibroblasts is capable of altering certain characteristics of both the dermis and epidermis. Differences between volar and nonvolar skin have been well-documented. The volar epidermis is thicker, has higher KRT9 concentrations, and larger cells (Vela-Romera et al., Fu et al.). The volar dermis has longer collagen fibers than the nonvolar dermis. Injection of fibroblasts from a volar dermis to a nonvolar dermis should be capable of modifying the skin to become more similar to the native volar environment. In fact, volar fibroblast injections appear to be capable of increasing epidermal thickness, KRT9 expression, and dermal collagen length. While these changes may be

observed, the mechanism by which this occurs in uncertain and may be elucidated by additional information on the genes being expressed after injection.

It is still unclear how gene expression is affected by the injection of fibroblasts from different tissue sites. As a part of the ongoing project on the effect of fibroblast transplants on epidermal identity, changes in gene expression following fibroblast injection were analyzed. There were two questions of particular interest: what effects fibroblasts have compared to vehicle injection, and what effect fibroblasts from the sole (volar fibroblasts) have compared to fibroblasts from the scalp (nonvolar fibroblasts). These questions were investigated using bioinformatic analysis of bulk and single cell RNAseq data from human tissue samples.

2.2 Methods

Fibroblast Preparation

Skin tissue biopsies were taken from the scalp and sole of two human subject groups. Each group contained three patients for a total of six patients. Samples were sent to the Hopkins Cell Center at the Genetics Resources Core Facility for cell expansion. Dermal fibroblasts were established in culture over the course of 8-10 weeks. Aliquots of 5 x 10^6 cells were cryopreserved and returned by the Cell Center.

Skin Injection and Biopsy

Cells were thawed immediately prior to injection. Each patient received dermal injections of their own cells at three adjacent dorsal sites, with one site receiving a vehicle, one site receiving the sole cells, and the other receiving scalp cells. One group (n=3) was given single injections of 10^6 cells at the experimental sites. The other group (n=3) received a series of three injections at each site, totaling 10^6 cells at each experimental site. The skin at the injection sites was biopsied 5 months after injection.

scRNAseq Processing

Extracted cells from a single patient (n=1) were sent to the Genetics Resources Core Facility for processing with the 10x Genomics Chromium Platform. Each cell was encapsulated within gel beads containing a unique 10x oligonucleotide barcode. Reverse transcription was done in all beads to generate cDNA, such that all cDNA from one cell shared the same 10x barcode. Sequencing was performed by the facility, and the data was sent back.

scRNAseq Analysis

The Seurat package (ver. 4.0) was used in the analysis of single cell RNAseq data (Hao et al., 2020). Pre-processing, normalization, and cell clustering was performed by Dr. Sam (Seakwoo) Lee. Basal keratinocyte populations 5 and 6 were identified as having particular differential expression, and were analyzed further.

RNA Extraction

RNA was isolated from the samples using the RNeasy MiniKit from Qiagen. Tissue samples were thawed and homogenized in 600 µl Buffer RLT with β-ME. Samples were centrifuged for 3 minutes at maximum speed, and the supernatant was removed. 70% ethanol was added to the supernatant. 700 µl of this sample was transferred to a spin column and centrifuged for 15 seconds. The column was rinsed once with Buffer RW1 and twice with RPE. RNase-free water was added to the column to elute RNA. Extracted RNA was sent to the Genetics Resources Core Facility for sequencing.

Bulk RNAseq Analysis

Paired-end reads from the RNAseq data were aligned by Dr. Sam (Seakwoo) Lee with the human reference genome, hg38 using STAR, a splice-aware alignment tool. STAR mapped the RNA-seq data to the reference genome by identifying seeds, generating genome indices, and

aligning the reads. DESeq2 was used by Dr. Lee for differential gene expression analysis with an adjusted p-value below 0.05. Differentially expressed genes were identified between the sole and vehicle, and between the scalp and vehicle. Gene ontology analysis was performed using Cluster Profiler (Bioconductor R package) and web-based David informatics (https://david.ncifcrf.gov/). Figures were generated using the GOplot package (ver. 1.0.2) in R.

2.3 Results

Bulk RNAseq

The results of gene ontology analysis indicate a clear, albeit unexpected, difference between the scalp (nonvolar) and sole (volar) fibroblast sites and the vehicle-injected site. While the differentially expressed terms were not the ones that would have been anticipated, there were a number of significantly differentially expressed terms in both the volar and nonvolar injection sites ($p<0.0002$). Due to the use of DESeq2 during data processing, the GO enrichment results show the differences between the fibroblast-injected sites and the vehicle-injected control site (Figure 1). The nonvolar and volar sites have many GO terms in common, mostly relating to metal ion transport, ion transmembrane signaling, and regulation of membrane potential (Figure 1). Some of the highly enriched soles exclusive to the volar site were associated with cell-cell and cell-matrix adhesion (Figure 1). Overall, the fibroblast-injected sites exhibited differential expression of genes associated with ion transport, signaling, and adhesion compared to the vehicle.

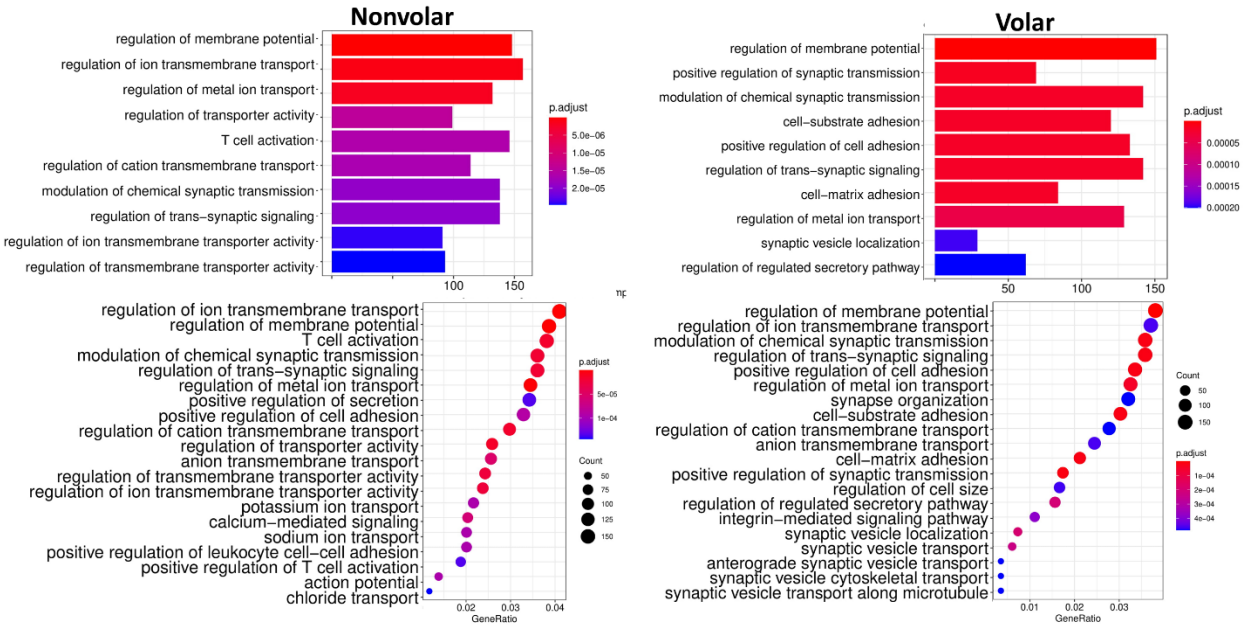


Figure 1: Gene Ontology Term Enrichment at Volar and Nonvolar Injection Sites. Gene ontology analysis only included genes with differential expression from the vehicle injection site. Top GO terms at the volar site are associated with cell size and cell adhesion, while top terms at the nonvolar site are associated with ion transport

To further investigate the difference between the volar and nonvolar injection sites, the regulation of individual genes was investigated. The data given by the GOplot in Figure 1 indicates which terms have the greatest change in gene expression, but not the direction in which gene expression changed. Using circle plots, it is possible to visualize each gene associated with the top GO terms to see if it was upregulated or downregulated (Figure 2). Amongst the shared GO terms, such as “Synaptic Membrane” and “Postsynaptic Membrane”, gene expression was relatively similar (Figure 2). Expression appears somewhat increased for genes associated with cell-substrate adhesion in the volar-injected sample (Figure 2).

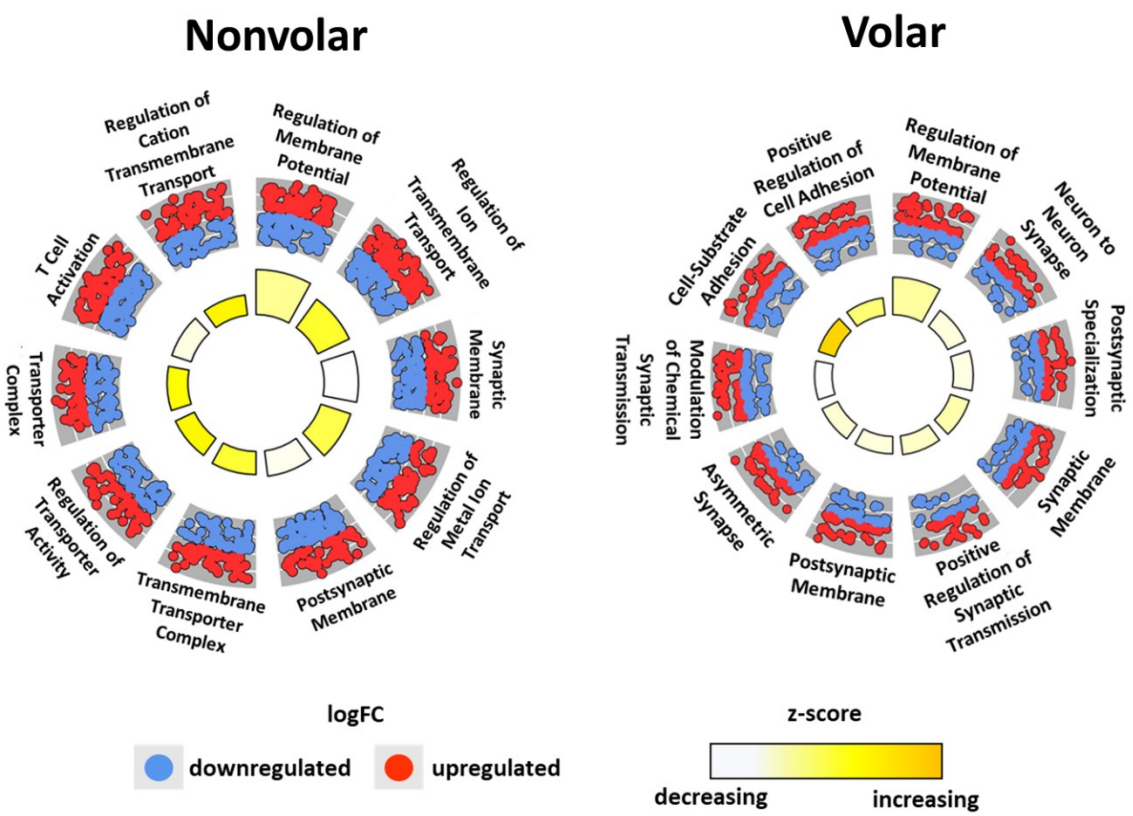


Figure 2: Gene Expression Levels at Volar and Nonvolar Injection Sites.
Gene expression levels for the top 10 enriched GO terms indicate whether each function is increased or decreased at the injection site. A high number of terms associated with synapses are present at each site, with similar levels of expression. Cell adhesion terms are slightly upregulated at the volar injection site.

scRNAseq

Clustering of the integrated Seurat object with principal component analysis yielded 17 distinct clusters. Among those clusters, basal keratinocyte populations 5 and 6 (BAS5/6) showed the greatest variation between vehicle, scalp, and sole fibroblast injected samples (Supp. Figure 1). These clusters were identified as a possible transitional population, and gene ontology analysis of these populations illustrated significant alterations to gene expression. As in the bulk RNAseq data, GOplots for identifying differences between the fibroblast-injected sites and the control site. Within the BAS6 population in both the volar- and nonvolar-injected sites, epidermal cell differentiation and skill development terms were enriched (Figure 3). This trend

was also present in the BAS5 population that received volar injections (Figure 3). These terms were not enriched in the BAS5 population at the nonvolar injection site, although regeneration and response to wounding were enriched (Figure 3). GO terms related to development, differentiation, and regeneration were enriched in the fibroblast-injected samples compared to the vehicle-injected sample.

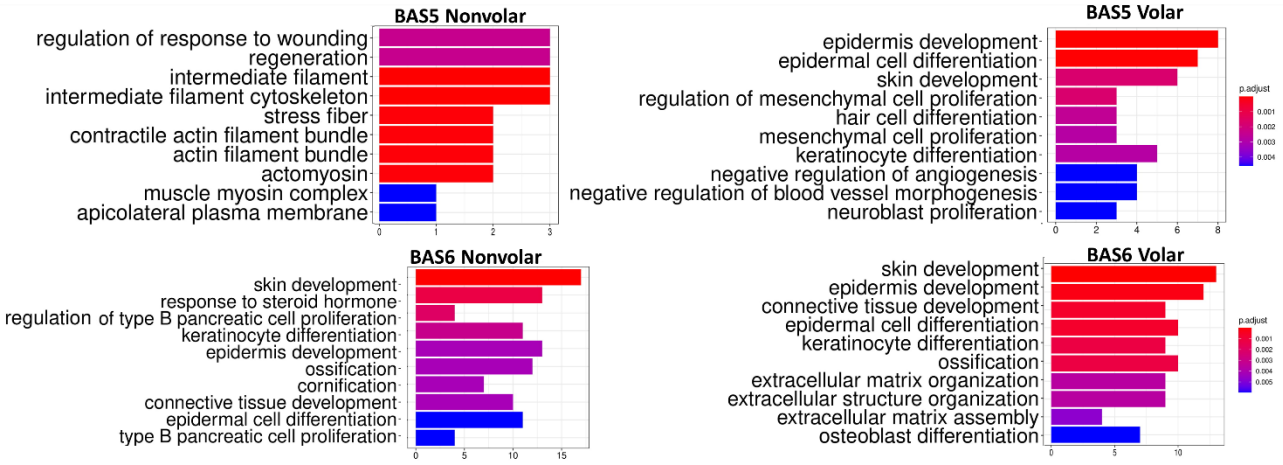


Figure 3: Gene Ontology Term Enrichment Within Basal Keratinocyte Populations
Gene ontology results demonstrated the most enriched terms at the volar and nonvolar injection sites within the BAS5/6 keratinocyte populations. Similar terms are enriched across all samples, with skin development, epidermal development, and epidermal cell differentiation being prominent across these populations.

Differences between volar and nonvolar fibroblast injections were compared again using circle plots. Within the BAS6 population, genes associated with skin development and differentiation were mostly downregulated in the nonvolar site, while these genes tended to be upregulated at the volar injection site (Figure 4). Genes associated with these terms were also upregulated in the BAS5 population at the volar injection site, while genes associated with regeneration were somewhat upregulated at the nonvolar injection site in this population (Figure 4). Overall, it appears that while fibroblast injections affected the same GO terms at both sites, upregulation of genes was more common following volar fibroblast injection, while downregulation of genes was more common following nonvolar injections.

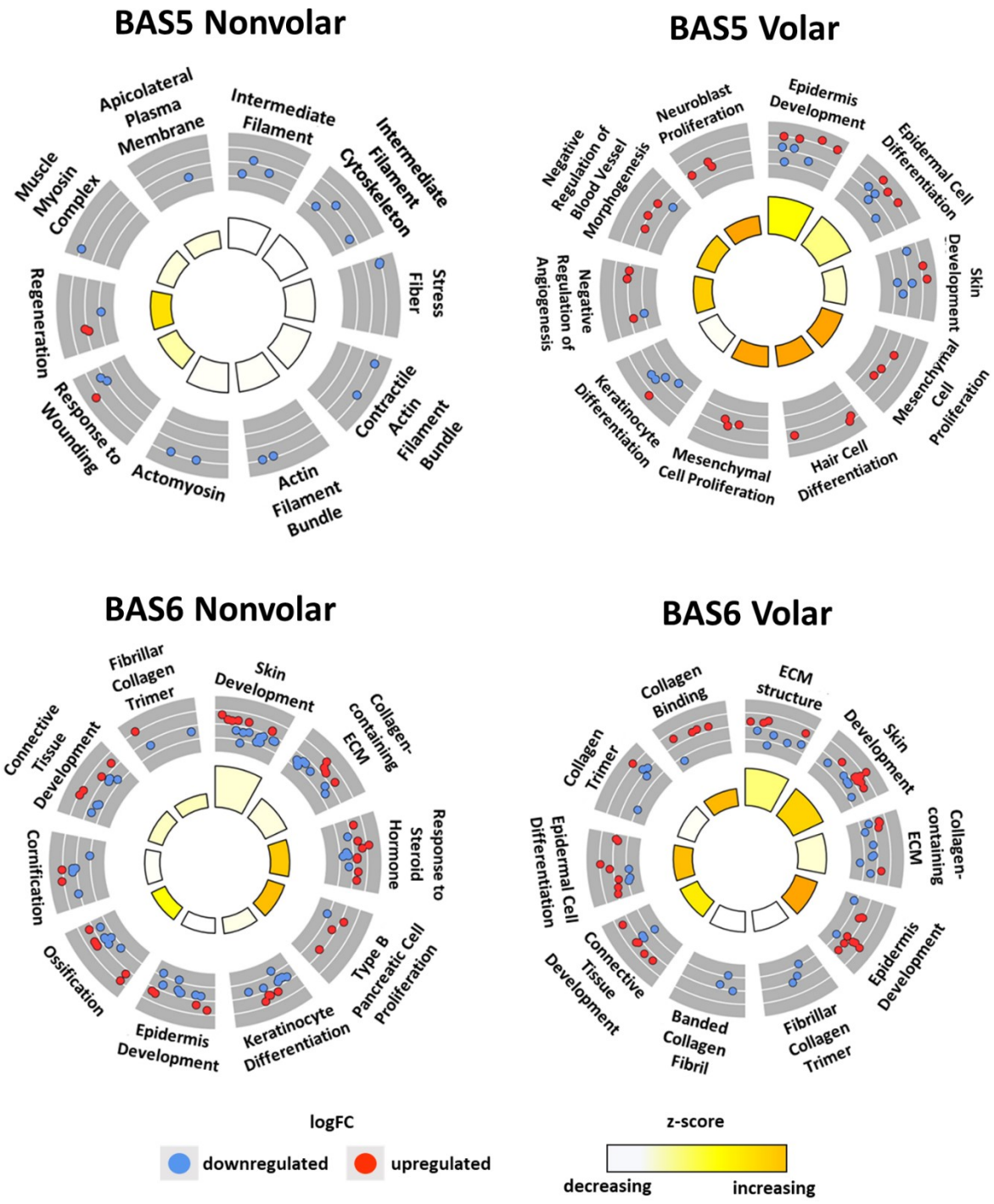


Figure 4: Gene Expression Levels Within Basal Keratinocyte Populations
Gene expression levels for the top 10 enriched GO terms indicate whether each function is increased or decreased within the BAS 5/6 keratinocytes at the injection site. Cell differentiation and development was upregulated in the populations at the volar injection cite. These terms were downregulated at the nonvolar injection site, although regeneration was slightly upregulated.

2.4 Discussion

The observed modifications to gene expression are indicative that the injected fibroblasts may be altering the epidermal identity. This was most obvious in the scRNA-seq data, but is also indicated in the bulk RNA-seq data. The increase in terms associated with skin development and epidermal differentiation in the scRNA-seq data clearly shows that the skin identity is beginning to change compared to the control. There are two conclusions that may be drawn from these results: firstly, that the injection of fibroblasts from either the sole or the scalp is capable of altering the expression of multiple genes, and secondly, that the injection of sole fibroblasts is capable of upregulating differentiation. This may be indicative of the sole fibroblasts converting the epidermis to be more similar to native sole.

The results of the bulk RNAseq are more difficult to interpret due to the similarities between the volar and nonvolar injection sites, but are at least indicative of the fibroblasts having some effect beyond that of the vehicle. This is not entirely unexpected, as bulk RNAseq measures the average RNA levels across all cell types in a tissue sample. scRNAseq separates the RNA expression levels by cell type, allowing for further investigation into RNA levels in similar cell types. In this way, scRNAseq provides a more refined analysis of expression, while certain information may be obscured in bulk RNAseq analysis. It may also be likely that the sole fibroblasts had a greater effect than the scalp fibroblasts. Fibroblasts play an important role in the stem cell niche; thus, they may have a large impact on their surrounding tissue (Boehnke et al.). Epidermal stem cells are heavily influenced by cell-cell and cell-matrix interactions, two terms that were highly enriched at the volar injection site (Boehnke et al.). While more subtle, this difference could indicate a substantial effect being had by sole fibroblasts. A difference that

appears small in bulk RNA-seq may prove be quite a bit more significant after looking closer into the most affected cell type, basal keratinocytes in this case.

The results of this experiment are significant as they provide insight into the changes to gene expression that occur following injection of fibroblasts from other skin types. Looking into these changes provides information on whether or not a treatment is doing what it is expected to do. More importantly, understanding the changes to gene expression that occur throughout the whole tissue allow for a more precise identification of targets for additional experimentation. By identifying the most important cell clusters, it is possible to target therapies more directly to those cells. Additionally, information about overall gene expression can assist in the identification of pathways to target. Within this experiment, possible pathways to investigate further are the Wnt pathway and the EGFR pathway, both of which are involved in epidermal differentiation and development (Boehnke et al.).

Chapter 3: Analysis of Gene Expression in Epidermal Wound Healing

3.1 Introduction

Wound healing is a complex process that is not yet entirely understood. The stages of wound healing are well documented, but there has not been much research into the differences that occur at different locations on a wound. Older theories suggest that the wound edge may play a large role in wound healing. The absence of neighboring cells along the edge may trigger epidermal cell migration and proliferation (Singer & Clark). With single cell analysis, further advancements have been made in understanding the differences between normal and wounded skin. Wounding generally decreases the number of epithelial cells, but increases immune and fibroblast populations (Haensel et al.). There also appears to be increased TNFa signaling, hypoxia, EMT, and a5-integrin signatures within wounded skin (Haensel et al.). Wounded samples also exhibit greater levels of cell division, protein translation, and RNA splicing (Haensel et al.).

While these differences between wounded and unwounded skin are becoming clearer, it is not yet understood what differences exist between the center and edge of wounds. In order to better understand the wound healing process for use in regenerative medicine, it will be helpful to understand what changes occur in different regions of the skin around the wound. It is expected that there would be decreased keratinocyte populations and increased immune populations in response to wounding. Further, it is expected that wound healing will have occurred to a greater extent on the edge of the wound as compared to the center. To investigate the changes to gene expression in response to wounding, bioinformatic analysis was conducted using scRNAseq data from mice.

3.2 Methods

Mouse Wounding

1 cm^2 wounds were made on the backs of mice (n=5). These mice were left until the wound closed and scab formation began. Once the scab detached from the wounded area, 6mm punch biopsies were collected at the center and edge of the wound. Biopsies were also taken from normal, unwounded tissue. The five samples at each site were pooled together. These samples were sent to the Genetics Resources Core Facility for 10x capture and sequencing.

scRNAseq Pre-Processing

Single cell RNAseq data was analyzed using the Seurat package (ver. 4.0) in R. Low count cells, multiplets, and dead cells were filtered out. Cells were then filtered based on count numbers, such that only cells with less than 5% mitochondrial counts, and between 200 to 2500 unique feature counts were kept to generate a Seurat object. Pre-processing resulted in 1041 cells from the center, 1223 cells from the edge, and 3360 cells from the unwounded sample. Individual Seurat objects were generated for the center, edge, and normal conditions, then merged to form a single Seurat object.

scRNAseq Analysis

Datasets were normalized and variable features were identified. Cell cycle genes were regressed out, and clusters were identified via integration. Conserved markers within each cluster and differentially expressed genes between clusters were identified from the original dataset. These markers were used to identify each cluster. Differentially expressed genes with an adjusted p-value below 0.05 were used in Gene Ontology analysis (Bioconductor R package).

3.3 Results

Cell Clustering

Clustering of the integrated mice cells yielded 16 distinct clusters. There were less cells overall in the wounded samples, which is unsurprising given that the wound has not yet fully healed (Figure 5). Additionally, there is a significant shift in macrophage populations following wounding. While the number of cells in this populations remains, macrophages shifted from population I to population II/III (Figure 5). T-cell populations remain relatively consistent across conditions. Finally, there is a significant decrease in keratinocyte and fibroblast populations after wounding. Surprisingly, there was a population of spinous keratinocytes present in the center of the wound that was not present in other conditions (Figure 5). Despite this abnormality, other keratinocyte and fibroblast populations followed the expected trend, with significantly fewer cells in the edge and almost none in the center of the wound (Figure 5).

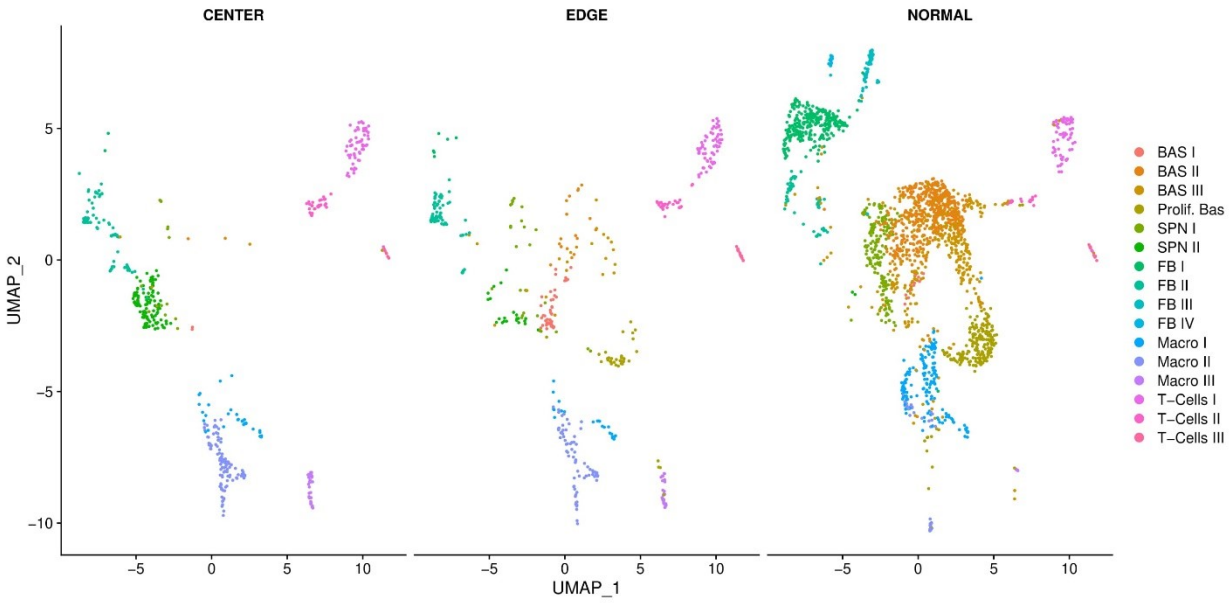


Figure 5: Cell Clustering of Wound Center, Wound Edge, and Normal Tissue
Fewer cells are present in wounded samples, while immune cell populations increase in response to wounding

Differential gene expression between conditions was also observed. Markers for cell clusters were investigated first to confirm the results of the dimensional plot (Figure 6). As expected, immune markers were upregulated in more cells throughout the wounded sites, while

proliferative basal keratinocyte marker, Mki67, was upregulated in more unwounded cells (Figure 7). Fibroblast markers, Col3a1 and Col1a2, were expressed in a similar number of cells in each condition, but were downregulated at the center of the wound (Figure 7). Surprisingly, keratinocyte markers were significantly upregulated in the center of the wound, despite the large decrease in keratinocyte population (Figure 7).

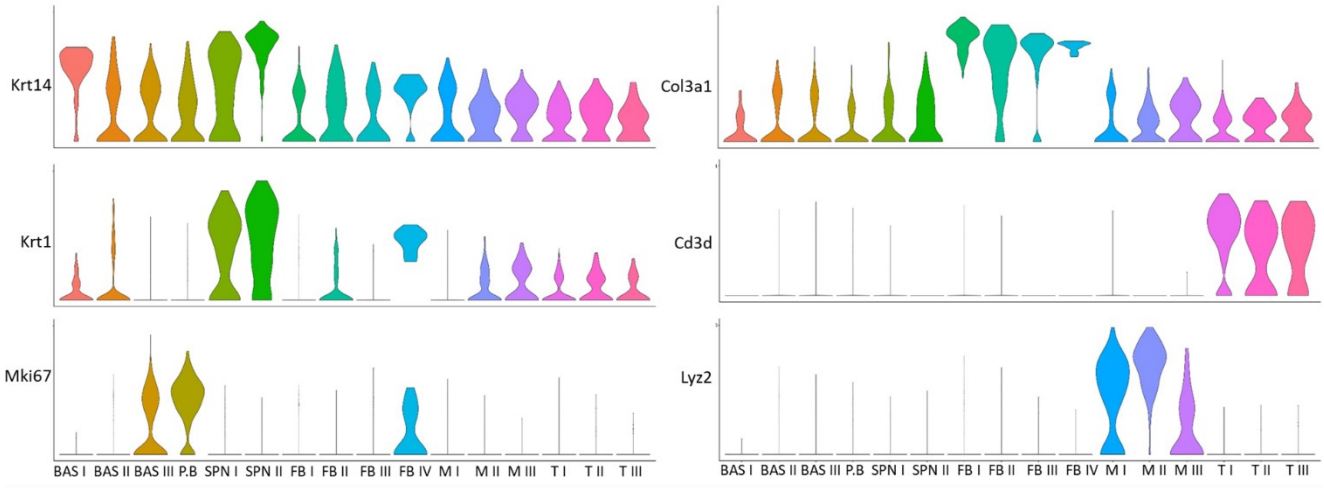


Figure 6: Cell Cluster Markers
Violin plots show expression of identified cell cluster markers in each cell population

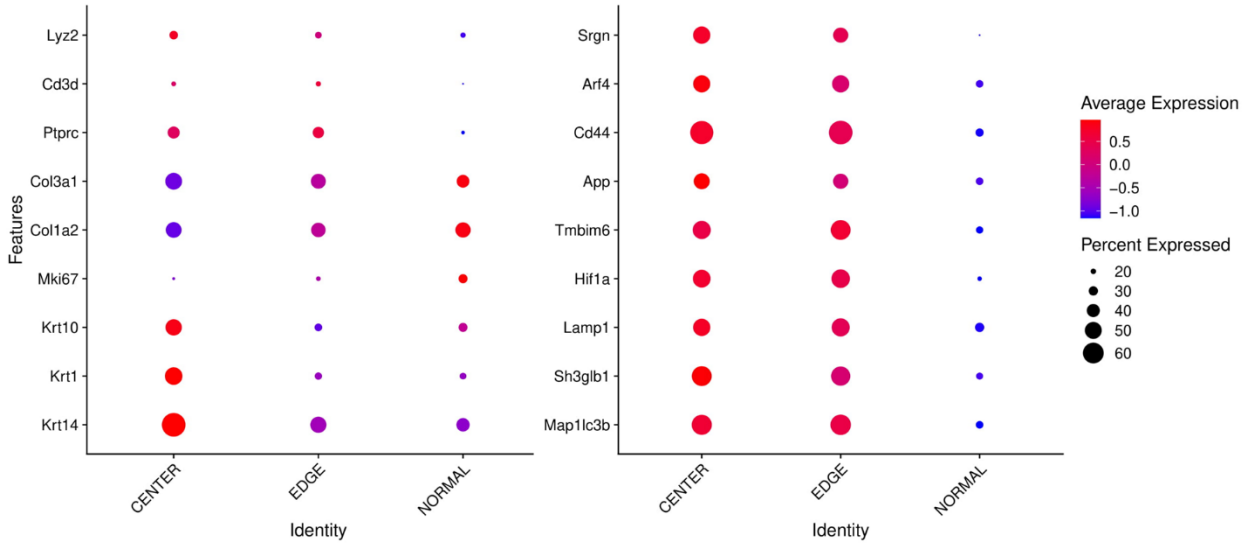


Figure 7: Gene Expression About Wound Site
Expression of gene cluster markers and genes associated with cell death or migration

Differentially expressed genes from the variable features of each Seurat object shed further light on differences that occur during wound healing. While gene expression was not significantly different between the center and edge, there were clear differences between wounded and unwounded skin. Genes associated with autophagic cell death (Lamp1, Sh3glb1, Map1lc3b, Tmbim6) and cell migration (Cd44, Arf4) were upregulated in a large number of cells in wounded tissue (Figure 7).

Gene Ontology

Gene ontology analysis revealed significant differences between wounded and unwounded skin, as well as differences between the center and edge of the wounded area. Overall, wounded tissue expressed a greater number of genes associated with the chemotaxis and migration of immune cells, including neutrophils, granulocytes, and leukocytes (Figure 8). The nearby unwounded tissue shows increased expression of T-cell, lymphocyte, and leukocyte differentiation, as well as increased leukocyte cell-cell adhesion (Figure 8). Additional differences exist between the center and edge of the wound. While the center exhibited increased translation, ribosome production, and ribosome assembly, the edge had an increased response to wounding including epidermal development, epithelial cell proliferation, and healing (Figure 8).

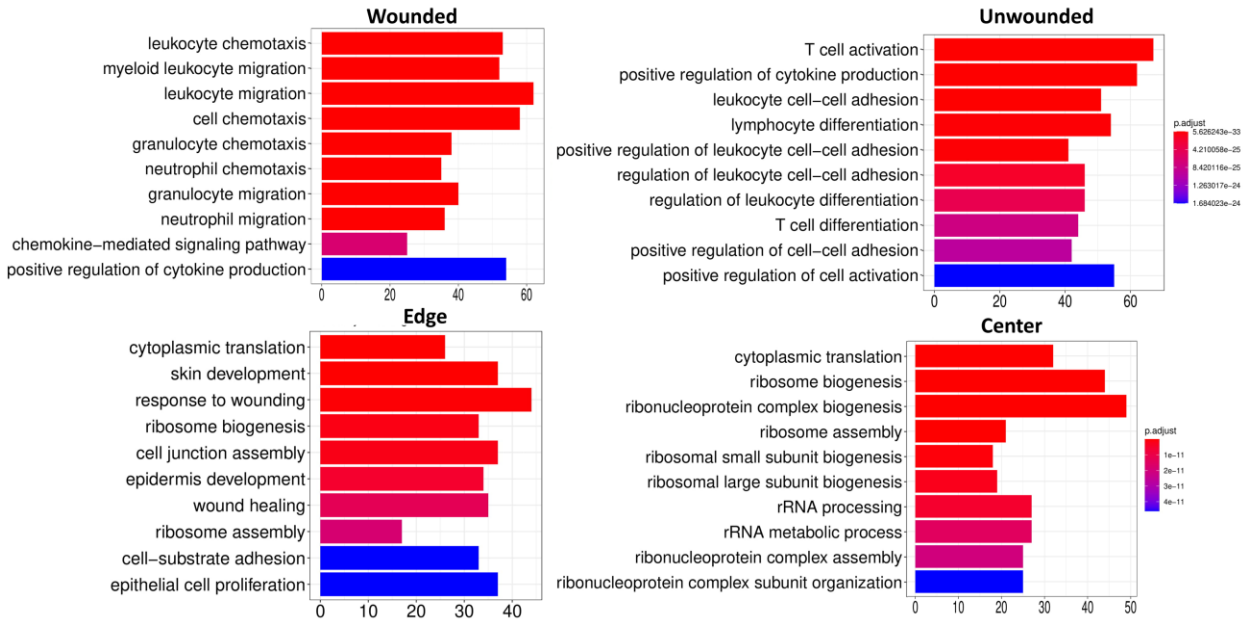


Figure 8: Gene Ontology Analysis of Wound Site
Top 10 terms associated with each condition

3.4 Discussion

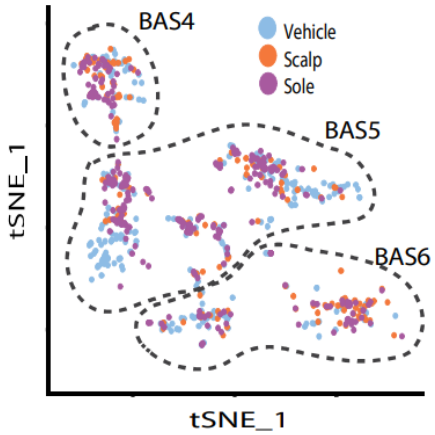
The results of scRNAseq analysis yield interesting insight into the differences that may occur at different wound sites throughout the process of healing. The results of cell clustering seem to agree with that found in other studies. The decrease in keratinocytes and increase in immune cells is reflective of normal healing processes. A strange observation was the increase in keratins in the wounded sample as there are decreased keratinocytes, thus it may be interesting to identify where these proteins are coming from, possibly by labeling Krt14 in different cell populations at the center and the edge.

Of additional interest is the observed difference between the center and edge of the wound. The gene ontology analysis indicates that gene expression is being differentially modified at the edge of the wound. The center of the wound has a significant increase in ribosomal assembly and translation, while the edge contributes more to tissue development. This is of interest because it contradicts the findings of another study that observed decreased

ribosomal activity in wounded skin (Haensel et al.). Given that this study only looked at wounded skin as a whole and did not differentiate by location, it is possible that ribosomal activity could be upregulated in the center of the wound but not around the edges.

Finally, these results are significant because they provide justification to further investigate how different areas of a wound alter. Much additional information can be gathered from this dataset, and the results presented above are preliminary findings. With this data, it will be possible to determine which genes and pathways are having the most effect in different areas of the wound and how cell populations change in response. This will allow for novel hypotheses on the topic of wound healing and regeneration.

Supplementary Information



Supplementary Figure 1: Basal Keratinocyte Clustering
Integrated tNSE plot of selected keratinocyte populations (BAS4-6) with differential distribution

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